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14. ABSTRACT
Although autism spectrum disorder (ASD) is defined by core behavioral impairments, gastrointestinal (GI) symptoms are commonly reported. Subsets of ASD individuals display dysbiosis of the gut microbiome, and some exhibit increased intestinal permeability. We demonstrate GI barrier defects in a mouse model of an important ASD risk factor, maternal immune activation (MIA). Remarkably, oral treatment of MIA offspring with the human commensal *Bacteroides fragilis* corrects gut permeability and ameliorates defects in communicative, stereotypic, anxiety-like and sensorimotor behaviors. MIA offspring also display an altered serum metabolomic profile, and *B. fragilis* normalizes levels of several of the serum metabolites. These findings suggest a gut-microbiome-brain connection in autism, and identify a potential probiotic therapy for ASD. We have now developed assays for some of these serum metabolites and are beginning to assay them in ASD serum samples

15. SUBJECT TERMS
autism, ASD, probiotic, maternal infection, microbiome, leaky gut

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Introduction

Autism is a neurodevelopmental disorder characterized by stereotypic behavior and deficits in language and social interaction. The incidence of autism has rapidly increased in the United States, representing a significant medical and social burden in the coming decades. However, therapies for treating the core symptoms of autism are limited, and reproducible molecular diagnostics have not been developed. Much research into autism spectrum disorder (ASD) has focused on genetic, behavioral and neurological aspects of the illness, but primary roles for environmental risk factors (Hallmayer et al., 2011), immune dysregulation (Onore et al., 2012) and additional peripheral disruptions (Kohane et al., 2012) in the pathogenesis of ASD have recently gained significant attention. Of several potential contributions to ASD, gastrointestinal (GI) distress is of particular interest, given its prevalence and correlation with the severity of core autism behavioral abnormalities (Bouie et al., 2010; Adams et al., 2011; Coury et al., 2012). A significant subset of ASD children display GI abnormalities, including abdominal cramps, chronic diarrhea or constipation and increased intestinal permeability (D'Eufemia et al., 1996; de Magistris et al., 2010). Moreover, antibiotic treatment and restricted diet are reported to provide behavioral improvements for some autistic children (Bouie et al., 2010).

The causes of these GI problems are unclear, but may be linked to gut bacteria, as the intestinal microbiome is altered in ASD individuals (Finegold et al., 2012; Williams et al., 2012). In animal models, the microbiota not only modulates the development and function of the enteric immune system, but also impacts neuroinflammation (Ochoa-Reparaz et al., 2010; Hooper et al., 2012). In particular, the human commensal *Bacteroides fragilis* exhibits therapeutic properties in mouse models of both colitis and multiple sclerosis (MS) (Ochoa-Reparaz et al., 2010; Round et al., 2010). Therefore, we are exploring the utility of commensal bacteria in treating ASD-like symptoms in an ASD mouse model.

Maternal immune activation (MIA) is an important environmental risk factor for ASD. Several large epidemiological studies link maternal viral and bacterial infection with increased autism risk in the offspring (Atladottir et al., 2010; Lee et al., 2012). We have shown that modeling this risk factor in mice by injecting pregnant dams with the viral mimic poly(I:C) yields offspring that exhibit the core behavioral symptoms of autism, as well as a common autism neuropathology (Shi et al., 2009; Malkova et al., 2012). MIA offspring also display altered peripheral immune responses (Hsiao and Patterson, 2011), which aligns well with recent studies highlighting a role for immune dysregulation in ASD (Onore et al., 2012). Although several environmental and genetic risk factors have been investigated in mice, GI abnormalities have not been reported in preclinical models of ASD.

Experimental Results Relevant for Tasks 1 and 2

In last year's summary we reported that the MIA offspring display increased GI epithelial permeability (Fig. 1A), as is found in significant fraction of human ASD cases. This is likely due to altered expression of several tight junction proteins (Fig. 1B). In addition to these changes, colonic tissues from adult MIA offspring display increased levels of interleukin-6 (IL-6) mRNA and protein (Figs. 1C, D) and decreased levels of the cytokines/chemokines IL-12p40/p70, IP-10, MIG and MIP-1a (Fig. 1D). Small intestines from MIA offspring also exhibit altered cytokine/chemokine profiles, characterized by elevations in FGF-basic, IL-1a, IL-2, IL-6, IP-10 and KC (data not shown). Changes in intestinal cytokines are not accompanied by overt GI pathology, as assessed by histological examination of gross epithelial morphology from hematoxylin- and eosin-stained sections (data not shown). Consistent with the alterations in immune-related signaling factors, however, mesenteric lymph nodes and spleens from adult MIA offspring contain decreased levels of regulatory T cells and hyper-responsive production of IL-6 and IL-17 by CD4+ T helper cells, suggestive of a pro-inflammatory phenotype (Hsiao et al., 2012). Similar findings supporting enteric immune activation are seen in subsets of ASD individuals (Onore et al., 2012). Overall, we demonstrate that adult offspring of immune-activated mothers exhibit increased gut permeability and abnormal intestinal cytokine profiles, recapitulating GI symptoms reported in ASD individuals in a mouse model. Moreover, as reported last year, a one-week treatment with *B. fragilis* at the time of weaning in MIA offspring restores GI permeability, tight junction components and GI

cytokines to normal levels, prevents the appearance of several bacterial metabolites in the serum, and prevents the development of ASD-like behaviors. We are currently developing assays for several of these metabolites so as to be able to measure their levels in human ASD serum. The goal of this part of the project is to examine whether these metabolites could be useful biomarkers for leaky gut and/or ASD.

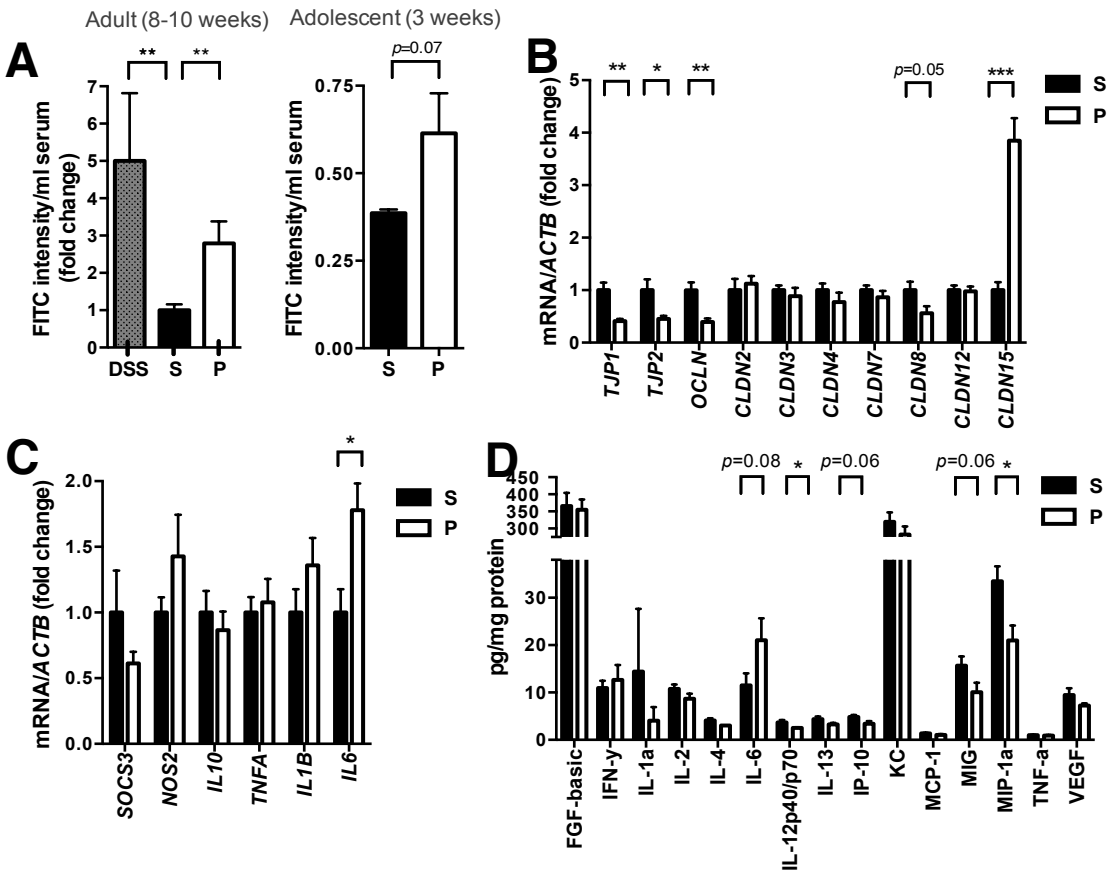

Figure 1. MIA offspring exhibit deficient GI barrier integrity and abnormal expression of tight junction components and cytokines. (A) Intestinal permeability assay, measuring fluorescence intensity of fluorescein isothiocyanate (FITC) detected in serum after oral gavage of FITC-dextran. DSS: n=6, S: adult n=16; adolescent n=4, P: adult n=17; adolescent n=4. Data are normalized to fluorescence intensity observed in adult saline offspring. (B) Expression of tight junction components relative to beta-actin in colons of adult saline and poly(I:C) offspring. Data for each gene are normalized to expression levels in saline offspring. n=8 (C) Expression of cytokines and inflammatory markers relative to beta-actin in colons of adult saline and poly(I:C) offspring. Data for each gene are normalized to expression levels in saline offspring. n=6-21 (D) Protein levels of cytokines and chemokines relative to total protein content in colons of adult saline and poly(I:C) offspring. n=10 Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. DSS=dextran sodium sulfate, S=saline+vehicle, P=poly(I:C)+vehicle. For each experiment, adult saline and poly(I:C) offspring were treated with vehicle at 3 weeks of age, and data were collected simultaneously for poly(I:C)+*B. fragilis* treatment group.

To begin to explore the mechanism of how leaky gut may be established in MIA offspring, we are examining the composition of the GI microbiota and the influence of probiotic treatment on it. We are also looking for parallels with findings that the GI microbiota is altered in human ASD. Numerous abnormalities related to the microbiota have been identified in autistic individuals, including enrichment in gut microbes such as *Clostridia*, *Desulfovibrio* and *Sutterella* (Adams et al., 2011; Finegold, 2011; Finegold et al., 2010; Finegold et al., 2012; Gondalia et al., 2012; Parracho et al., 2005b; Williams et al., 2011; Williams et al., 2012). To evaluate whether MIA induces microbiota alterations, surveyed the fecal bacterial population by high-throughput 16S rRNA gene sequencing in samples isolated from adult offspring of mothers treated with poly(I:C) or saline. Alpha diversity, i.e., species richness and evenness, did not differ significantly between control and MIA offspring, as measured by Faith's phylogenetic diversity (PD) index and the Gini coefficient (data not shown). Unweighted UniFrac analysis, which measures the degree of phylogenetic similarity between microbial communities, reveals a strong effect of MIA on the gut microbiota of adult offspring (Fig. 2). That MIA samples cluster distinctly from controls by principal coordinate analysis (PCoA) indicates robust changes in the membership of gut bacteria from MIA offspring compared to controls (Figure 2A). The effect of MIA on altering

the composition of the gut microbiota is evident when sequences from the classes Clostridia and Bacteroidia, which account for approximately 90% of the total reads in our survey, are exclusively examined by PCoA (Fig. 2B), but not when Clostridia and Bacteroidia sequences are specifically excluded from PCoA of all other bacterial classes (Fig. 2C). This indicates that changes in the diversity of Clostridia and Bacteroidia operational taxonomic units (OTUs) are the primary drivers of gut microbiota differences between MIA offspring and controls. Further stratifying Clostridia and Bacteroidia into the most abundant (>250 observations) and least abundant (<250 observations) OTUs reveals that PCoA clustering by treatment group is likely driven by changes in the abundance or detection of rare Clostridia and Bacteroidia OTUs (data not shown). 119 OTUs out of the 1859 OTUs detected across any of the samples discriminate between treatment groups, including those assigned to the bacterial families *Lachnospiraceae, Ruminococcaceae, Bacteroidaceae, Prevotellaceae* and unclassified *Bacteroidales* (Fig. 2D). Of these 119 discriminatory OTUs, 47 are more abundant control samples and 72 are more abundant in MIA samples. Consistent with the PCoA results (Figs. 2A-C), the majority of OTUs that discriminate MIA offspring from controls are assigned to the classes *Bacteroidia* (75 of 119 OTUs; 63.0%) and *Clostridia* (36 of 119 OTUs; 30.3%), whereas the few remaining discriminatory OTUs belong to *Proteobacteria* (3 OTUs; 2.5%) and other classes (5 OTUs; 4.2%). Interestingly, eight of the 14 discriminatory *Clostridial* OTUs identified as more abundant in control offspring are taxonomically classified to the order *Ruminococcaceae*, and 19 of the 22 discriminatory *Clostridial* OTUs identified as more abundant in MIA offspring are taxonomically classified to the order *Lachnospiraceae* (Fig. 2D). This suggests that specific *Lachnospiraceae* may play an important role in MIA pathogenesis, along with other species of the classes *Clostridia* and *Bacteroidia*. Importantly, there is no significant difference in the overall relative abundance of *Clostridia* and *Bacteroidia* between MIA offspring and controls (Fig. 2E, left panel), consistent with our finding that alterations in the membership of rare OTUs drive major changes in the gut microbiota between treatment groups.

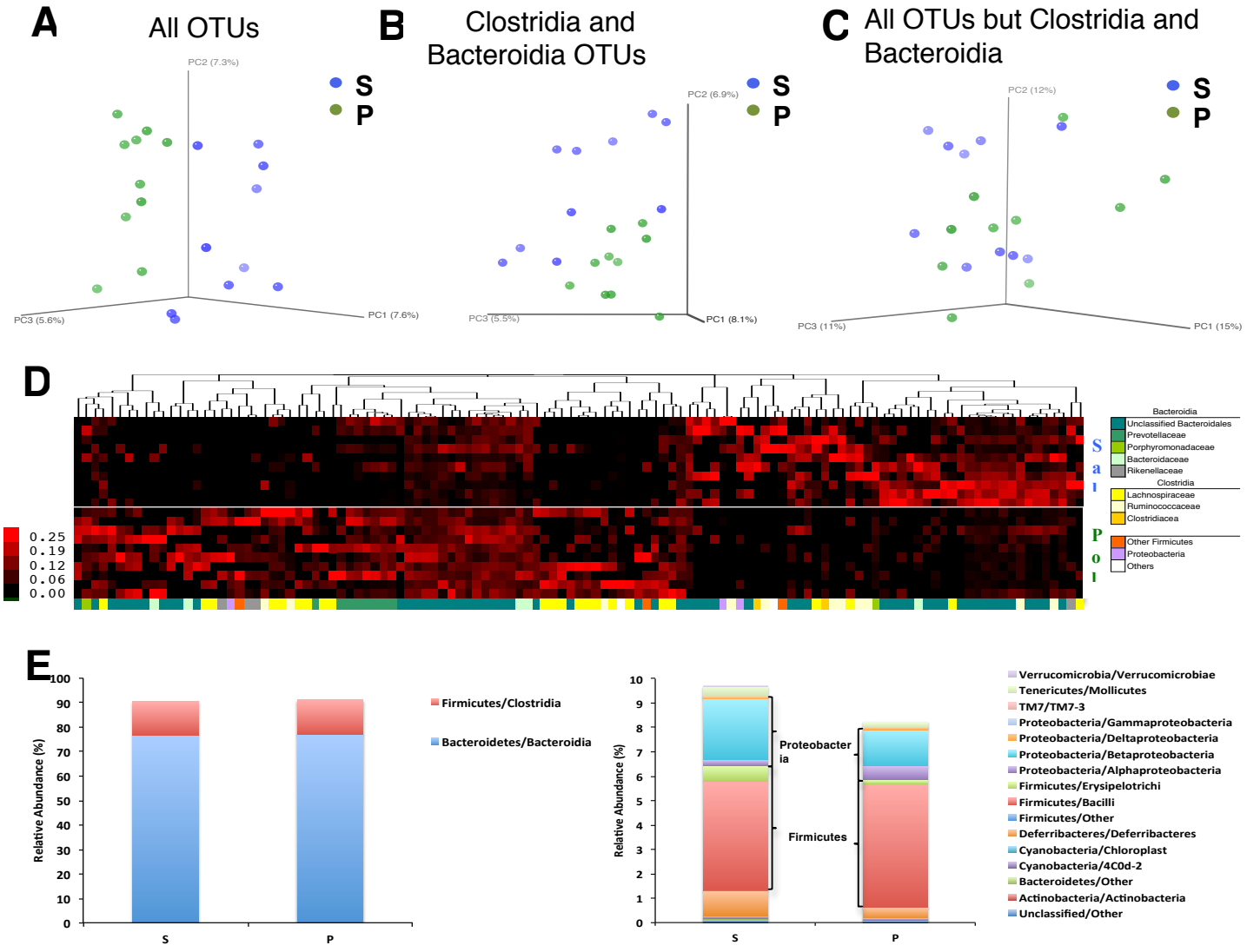

Figure 2. MIA offspring exhibit dysbiosis of the intestinal microbiota. (A) Unweighted UniFrac-based 3D PCoA plot based on all OTUs, illustrating global differences in the gut microbiota between adult MIA and control offspring. The percent variation explained by each principal coordinate (PC) is indicated on the axes. (B) Unweighted UniFrac-based 3D PCoA plot based on subsampling of *Clostridia* and *Bacteroidia* OTUs (2334 reads per sample). (C) Unweighted UniFrac-based 3D PCoA plot based on subsampling of OTUs remaining after subtraction of *Clostridia* and *Bacteroidia* OTUs (75 reads per sample). (D) Heat-map showing the relative abundance of unique OTUs of the gut microbiota (bottom, x-axis) for individual biological replicates from adult saline and poly(I:C) offspring (right, y-axis), where red of increasing intensity denotes increasing relative abundance of a unique OTU for a particular sample. All OTUs that significantly discriminate between treatment groups are plotted ($p < 0.05$, two-tailed Student's t-test or two-tailed Mann-Whitney test with Gaussian approximation when OTUs are absent from all samples)**.** Family-level taxonomic assignments as designated by the Ribosomal Database Project are indicated for each OTU. (E) Mean relative abundance of OTUs classified by taxonomic assignments at the class level for the most abundant taxa (left) and least abundant taxa (right). n=10. Data were simultaneously collected and analyzed for poly(I:C)+*B. fragilis* treatment group. See also Figure S3.

Differences in taxonomic diversity can be also seen in less prominent bacterial classes, with MIA offspring displaying significantly decreased relative abundance of *Erysipelotrichi* (0.16 ± 0.03 % v.s. 0.65 ± 0.23 %; mean ± SEM), *Betaproteobacteria* (1.48 ± 0.29 % v.s. 2.49 ± 0.49 %) and *Mollicutes* (0.21 ± 0.05 % v.s. 0.40 ± 0.14 %) and significantly increased relative abundance of *Alphaproteobacteria* (0.58 ± 0.27 % v.s. 0.24 ± 0.11 %) and other unclassified bacterial taxa (0.82 ± 0.51 % v.s. 0.12 ± 0.03 %), compared to controls (Fig. 2E, right panel).

Overall, we find that MIA leads to dysbiosis of the gut microbiota, driven primarily by alterations in specific OTUs of the bacterial classes *Clostridia* and *Bacteroidia*. Changes in OTUs classified as *Lachnospiraceae* and *Ruminococcaceae* of the order *Clostridiales* parallel reports of increased Clostridium species in the feces of subjects with ASD (Finegold et al., 2012). Altogether, modeling MIA as a primary autism risk factor in mice induces not only behavioral and neuropathological features of ASD (Boksa, 2010), but also GI symptoms analogous to those described in subsets of ASD individuals. This supports the utility of MIA as a relevant model for human ASD with comorbid GI issues.

In addition to ameliorating GI physiology in MIA offspring, *B. fragilis* treatment induces long-term effects on the composition of the intestinal microbiota. No significant differences are observed at the global level by PCoA or in microbiota richness and evenness (Fig. 2A). However, corrective effects of *B. fragilis* treatment are apparent upon evaluating specific key OTUs that discriminate MIA offspring from controls (Figs. 2B, C). Specifically, adult MIA offspring treated with *B. fragilis* display complete restoration in the relative abundance of 16 out of the 119 OTUs found to be discriminatory between MIA and control offspring, which are taxonomically assigned as unclassified Bacteroidia and Clostridia of the family Lachnospiraceae (Figs. 2B, C). Notably, these alterations occur in the absence of persistent colonization of *B. fragilis*, which remains undetectable in fecal and cecal samples isolated from treated MIA offspring, as assessed by quantitative real-time PCR (data not shown). Interestingly, eight of the 19 *Lachnospiraceae* elevated in MIA offspring are corrected by *B. fragilis* treatment (Fig. 2D and Fig. 3). In addition, *B. fragilis* treatment restores the relative abundance of eight *Bacteroidia* OTUs to levels observed in controls (Figs. 3B, C). Phylogenetic reconstruction of the 16 OTUs that are altered by MIA and completely restored by *B. fragilis* treatment reveals that all eight Bacteroidia OTUs cluster together into a monophyletic group (Fig. 3D). In addition, six of the eight Lachnospiraceae OTUs that are significantly altered by MIA and corrected by *B. fragilis* also cluster into a monophyletic group (Fig. 3D). This suggests that, though treatment of MIA offspring with *B. fragilis* may not lead to persistent colonization of *B. fragilis* itself, it appears to correct dysbiosis of specific groups of related microbes of the *Lachnospiraceae* family of *Clostridia* and unclassified or *Bacteroidaceae* family of *Bacteroidia*. That the restoration of *Bacteroidia* OTUs by *B. fragilis* treatment involves increases in particular OTUs and decreases in others suggests that *B. fragilis* treatment may favor specific bacterial species over their harmful relatives (Fig. 3D, compare purple OTUs to cyan OTUs). Altogether, we demonstrate that treatment of MIA offspring with *B. fragilis* ameliorates particular changes involved in MIA-associated dysbiosis of the commensal microbiota and corrects GI abnormalities analogous to those observed in subsets of autistic individuals.

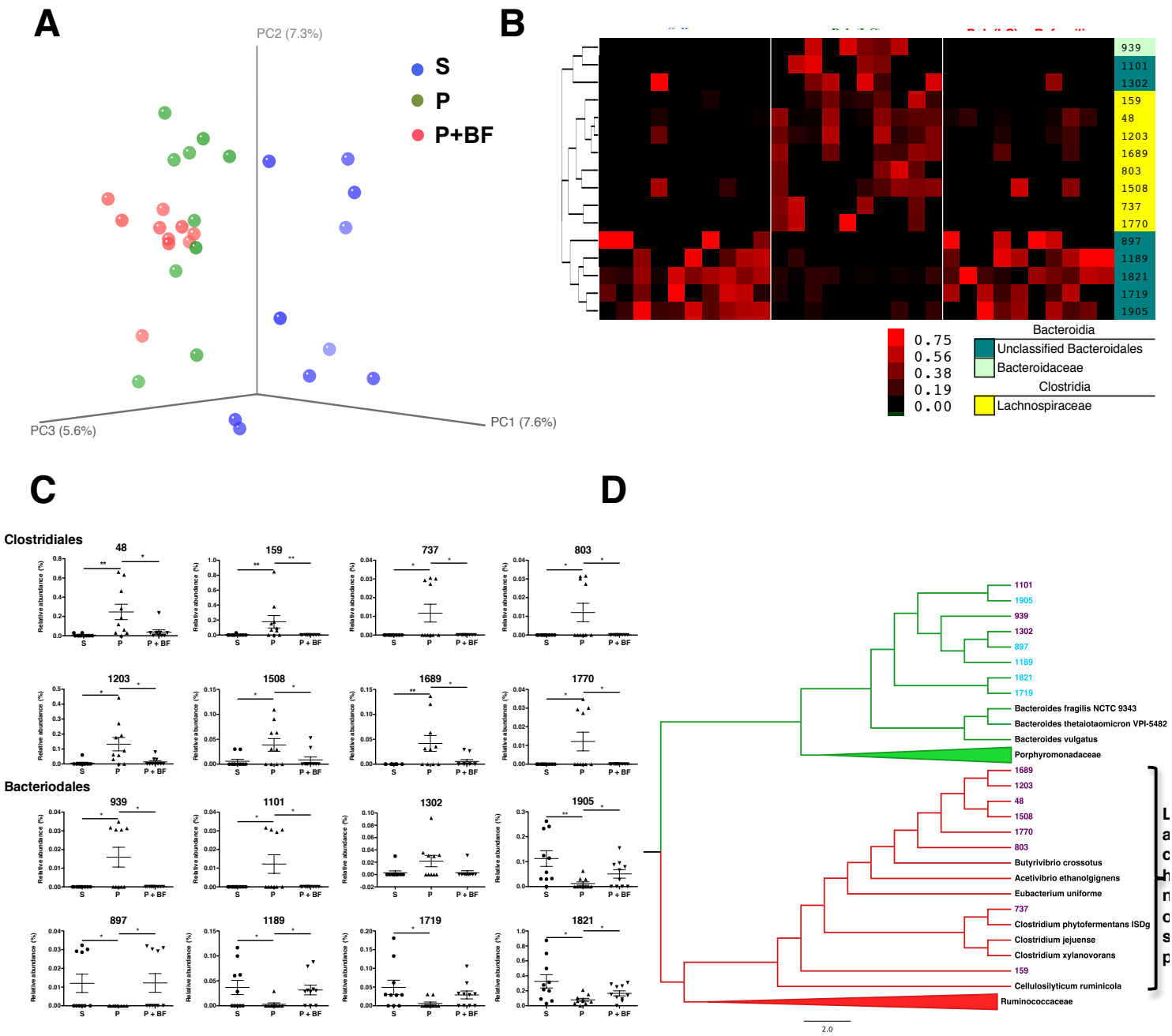

Figure 3. *B. fragilis* treatment alters the composition of the intestinal microbiome and corrects species-level abnormalities in MIA offspring. (A) Unweighted UniFrac-based 3D PCoA plot based on all OTUs. The percent variation explained by each principal coordinate (PC) is indicated on the axes. Data for saline and poly(I:C) are as in Figure 2. (B) Heat-map showing the relative abundance of key OTUs of the gut microbiota (right, y-axis) that are significantly altered by MIA and completely restored by *B.* fragilis treatment, where red of increasing intensity denotes increasing relative abundance of a unique OTU for a particular sample. Family-level taxonomic assignments as designated by the Ribosomal Database Project are indicated for each OTU. (C) Relative abundance of key OTUs of the order *Clostridiales* (top) and *Bacteroidales* (bottom) that are significantly altered by MIA and completely restored by *B. fragilis* treatment. Data are presented as mean ± SEM. **p* < 0.05, ***p* < 0.01. (D) Phylogenetic tree based on nearest-neighbor analysis of 16S rRNA gene sequences for key OTUs presented in panels B and C. Red clades indicate OTUs of the class *Clostridiales* and green clades indicate OTUs of the class *Bacteriodales*. Green wedge denotes combined members of the family Porphyromonadaceae. Red wedge denotes combined members of the family *Ruminococcaceae*. Purple taxa indicate OTUs that are significantly elevated in poly(I:C) offspring and corrected by *B. fragilis* treatment, whereas cyan taxa indicate OTUs that are significantly reduced in poly(I:C) offspring and corrected by *B. fragilis* treatment. n=10.

Key Research Accomplishments

- As in a very significant subset of ASD cases, the offspring of immune-activated pregnant mice display a leaky gut phenotype.
- Both the leaky gut and most of the ASD-like behaviors in these offspring can be prevented by administration of a probiotic bacterium.
- MIA offspring also display altered serum metabolites, some of which are corrected by probiotic treatment.
- The gut microbiome in the MIA model displays dysbiosis and analysis of the changes reveals striking parallels with microbiome abnormalities in human ASD.

Reportable Outcomes

Garay PA, Hsiao EY, Patterson PH, McAllister AK (2013) Maternal immune activation causes age- and region-specific changes in brain cytokines in the offspring throughout development. *Brain Behav Immun* 31:54-68. PMID 2241693

Hsiao EY, McBride S, Chow J, Mazmanian SK, Patterson PH (2012) Modeling an autism risk factor in mice leads to permanent immune dysregulation. *Proc Natl. Acad Sci USA* 109:12776-81. PMID 22802640 *[Highlighted with commentary; cited by the Autism Speaks and Simons foundations as one of the top 10 research advances in autism in 2012]*

Hsiao EY, McBride SW, Hsien S, Sharon, G, Alicki ER, McCue T, Codelli JA, Chow J, Reisman SE, Petrosino J, *Patterson PH, *Mazmanian SK. The gut microbiome modulates gut physiology and behavioral abnormalities in a mouse model of autism. Submitted to *Cell*. *contributed equally

Conclusions

While the impact of the microbiota on immunologic and metabolic disease is profound, little is known regarding a link to behavioral disorders. We find that commensal bacteria of the microbiota can influence the gut-brain connection by modulating metabolites that alter behavior. Postnatal *B. fragilis* treatment corrects abnormal GI permeability and ameliorates communicative, stereotyped, sensorimotor and anxiety-like behavior in a mouse model of an ASD risk factor. Our findings represent the first evaluation of a microbial effect on core autism-related behaviors. In addition to displaying cardinal behavioral and neuropathological symptoms of ASD, MIA offspring exhibit altered serum metabolites and deficient GI integrity that is analogous to that seen in subsets of ASD individuals (Altieri et al., 2011). Thus, the MIA model exhibits face and construct validity for particular co-morbid GI symptoms found in human autism. Consistent with the well-established role of GI microbes in regulating intestinal permeability and metabolic homeostasis, we show that *B. fragilis* treatment corrects GI permeability and restores MIA-associated changes in blood metabolites. By this means, *B. fragilis* may prevent the leakage of deleterious molecules from the GI lumen and/or promote the synthesis of protective compounds in the periphery. It is intriguing that *B. fragilis* exerts beneficial behavioral and metabolomic effects in a disease-specific manner. Taken together, we suggest a novel mechanism by which *B. fragilis* treatment can improve autism-related behavioral abnormalities and present compelling evidence for a probiotic-based therapy for ASD-associated symptoms. Validation of a specific metabolomic profile specific in human autism subjects with GI complications may serve as a novel molecular diagnostic and the basis for microbiome-mediated therapies.

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